Contents

Introduction

E.Z.N.A.® Tissue RNA Kit is designed for isolation of total RNA from animal tissues specially for some difficult fibrous tissues such as skeletal muscle, heart and aorta tissue. Those tissues normally make RNA isolation more difficult because they contains contractile proteins, connective tissue and collagen. E.Z.N.A® Tissue RNA Kit allows simultaneous processing of multiple tissue samples in less than 60 min The procedure completely removes contaminants and enzyme inhibitors making RNA isolation fast, convenient, and reliable. RNA purified using the E-Z 96® Tissue RNA method is ready for applications such as RT-PCR*.

Principle

The E.Z.N.A.® Tissue RNA Kit uses reversible binding properties of HiBind® matrix, a new silica-based, time saving spin technology material. The sample is lysed first under highly denaturing buffer conditions so that RNases will be inactivated, and the intact Tissue RNA is protected from degrading. After adjusting the buffer condition, the samples are treated with proteinase to remove proteins. Cell debris is pelleted by centrifugation. After adding ethanol to the cleared lysate, the sample is loaded to the HiBind® RNA Minicolumn. With a brief centrifugation or vacuum, the samples pass through the column and the RNA binds to the HiBind® matrix. Trace of the DNA that may co-purified with RNA will be removed by DNase treatment on the RNA spin column. After two washing steps, purified total RNA will be eluted with RNase-free water.

Storage and Stability

All components in the E.Z.N.A® Tissue RNA Kit should be stored at room temperature. OB Protease should be stored at 15-25°C. During shipping and storage, crystals may form in the TRK Lysis Buffer, simply warm to 37°C to dissolve. All kit components are guaranteed for at least 12 months from the date of purchase.

*The PCR process is covered by U.S. Patents 4,683,195 and 4,683,202 (and international equivalents) owned by Hoffmann-LaRoche, Inc.

Kit Contents

Product Number	R6688-00	R6688-01	R6688-02
Purification Times	5 Preps	50 Preps	200 Preps
HiBind™ RNA Mini column	5	50	200
2 ml Collection Tube	15	150	600
TRK Lysis Buffer	5 ml	20 ml	80 ml
RNA Wash Buffer I	5 ml	45 ml	2 x 90 ml
RNA Wash Buffer II Concentrate	2 ml	12 ml	4 x 12 ml
OB Protease	55 µl	550 µl	2 x 1.1 ml
DEPC Water	1 ml	10 ml	40 ml
Instruction Manual	1	1	1

Materials supplied by user

- 96-100% ethanol
- RNase-Free DNase I (optional)
- β-Mercaptoethanol
- RNase-free filter pipette tips
- Centrifuge capable of 14000 x g
- Water bath or heat block preset at 55°C

Before Starting

IMPORTANT

1. **RNA Wash Buffer II Concentrate** must be diluted with absolute ethanol (~96-100%)before use and store at room temperature.

   R6688-00 Add 8 ml ~96-100% ethanol

   R6688-01 Add 48 ml ~96-100 % ethanol

   R6688-02 Add 48 ml ~96-100% ethanol/bottle

2 **Optional :** Prepare the DNase I digestion mixture: For each RNA isolation, add 1.5µl of DNase I with 73.5µl DNase I digestion buffer.

Important Notes

Please take a few minutes to read this booklet thoroughly and become familiar with the protocol. Prepare all materials required before starting to minimize RNA degradation.

- Whenever working with RNA, always wear latex gloves to minimize RNase contamination. Use only clean RNase-free disposable plastic pipette tips when using the supplied reagents.
- During the procedure work carefully but quickly.
- Under cool ambient conditions, crystals may form in TRK Lysis Buffer. This is normal and the bottle should be warmed to redissolve the salt.
- 2-mercaptoethanol (ß-mercaptoethanol) is key in denaturing RNases and must be added to an aliquot of TRK Lysis Buffer before use. Add 20 µl of 2-mercaptoethanol per 1 ml of TRK Lysis Buffer. This mixture can be stored for 1 week at room temperature.
- All centrifugation steps must be carried out at 22°C-25°C.

Note: Equilibrate samples and TRK Lysis buffer to room temperature before start. All steps must be carried out at room temperature. Work quickly, but carefully.

Starting Material

Although the binding capacity for each well of the HiBind™ RNA column is around 100 µg, however, the maximum amount of starting material depend on the type of the tissue being processed and the corresponding RNA content. **It is essential to begin with correct amount of tissue to get optimal RNA yield and purity with HiBind™ RNA Minicolumn. For the first time user, we recommend to use less than 10 mg tissue. Depending on the yield and purity obtained, it may be possible to increase the starting material to 30 mg.**

Disruption & Homogenization of Tissues

A. Disrupt and Homogenize Tissue with Liquid Nitrogen

Wear gloves and take great care when working with liquid nitrogen. Excise tissue and promptly freeze in a small volume of liquid nitrogen. Grind tissue with a ceramic mortar and pestle under approximately 10 ml of liquid nitrogen and pour the suspension into a pre-cooled 15 ml polypropylene tube. Unless the tube is pre-cooled (in liquid nitrogen), the suspension will boil vigorously possibly causing loss of tissue. When the liquid nitrogen has completely evaporated, add TRK Lysis Buffer and continue with the procedure as outlined below. After interrupting tissue, lysate can be homogenized with Omega Homogenizer Spin Column (Product # HCR003). **The lysate is loaded onto Omega Homogenizer Spin Column in a 2 ml collection tube**. Spin two minutes at a maximum speed in a microcentrifuge and the homogenized lysate collected. Use the Omega Homogenizer Spin Column is fast and efficient way to homogenize the lysate without cross contamination of samples. The alternated way to homogenize the lysate is to use the syringe and needle. High molecular-weight DNA can be sheared by passing the lysate through a narrow needle (19-21 gauge) for 5-10 times.

B. Disruption and Homogenization with Rotor-Stator

Rotor-Stator is the most preferred method for disruption and homogenizing tissue samples if required equipments are available. Rotor-stator homogenizers effectively homogenize and homogenize most tissues in the present of TRK Lysis Buffer. The process usually takes less than a minute depending on the tissue. Many Rotor-stator homogenizers operate with differently sized probes or generators that allow processing of small volumes in microfuge tubes. Such homogenizers are available from:

- Tekmar Inc., Cincinnati, OH (Tissuemizers®)
- BIOSPEC Products, Bartlesville, OK (Tissue-Tearor™)
- Craven Laboratories, Austin, TX.

C. Disruption and Homogenization with Beads Mills

Tissue sample can also be effectively disrupted and homogenized by rapid agitation in the presence of beads and lysis buffer. Tissue samples are disrupted and simultaneously homogenized with the sheared and crushed action of the beads as they collide with cells.

E.Z.N.A.® Tissue RNA Protocol

1. **Excise the tissue sample from animal or from storage**.

2. **Weight 10-30mg tissue and place it into suitable vessel for disruption and homogenization.** *Do not use more than 30 mg tissue. For more information, see page 4 of Starting material.*

3. **Add 300 µl TRK lysis Buffer/2-ME and disrupt tissue and homogenize tissue in TRK lysis buffer**. Rotor-Stator or Beads Mills normally can result higher RNA yield because they provided better disruption and homogenization.

   Note:2-mercaptoethanol (ß-mercaptoethanol) is key in denaturing RNases and must be added to an aliquot of TRK Lysis Buffer before use. Add 20 µl of 2-mercaptoethanol per 1 ml of TRK Lysis Buffer. This mixture can be stored for 1 week at room temperature.

4. **Pipet 300 µl RNase-Free water to each homogenate and add 10 µl OB Protease and mix throughly by pipetting.** Incubate at 55°C for 10 minutes.

5. **Centrifuge at ≥14,000 x g for 3 minutes at room temperature**. A small pellet of tissue debris will form and a thin layer or film can be seen on top of the supernatant.

6. Transfer the supernatant into a RNase-Free 1.5 ml microcentrifuge tube. (Not provided).

   Note: Avoid to transfer any of pellet. Hold the pipett tip under the thin layer or film on top of the supernatant, if present. This layer will usually adhere to outside the tip and should not be transferred.

7. **Add 0.5 volume of absolute ethanol (96-100%) to the cleared lysate, mix throughly by pipetting.**

8. **Carefully apply 700µl samples from step 7 (including any precipitate) to HiBind® RNA Mini column in a 2 ml collection tube.** Centrifuge at 10,000 x g for 1 min at room temperature. Discard the flow-through and re-use the collection tube in step 9.

9. Repeat steps 8 to load the remaining of samples to the column. Centrifuge as above and discard the flow-through. Re-use the collection tube in step 11.

10. **Pipet 300µl RNA wash Buffer I into the column. Centrifuge as above and** Discards the flow-through. Re-use the collection tube in step 11. If the On-Membrane DNase treatment is not desired, simply increase the volume of RNA wash Buffer I to 700µl, centrifuge at 10,000 x g for 30 seconds to wash the column and discards the flow-through and collection tube. Continue the protocol with step 12.

11A. (**Optional): Prepare the DNase I mixture:** In a 1.5 ml microtube, add 1.5µl

DNase I to 73.5µl DNase I Digestion Buffer. Mix gently by inverting the tube. Do not vortex, DNase I is especially sensitive with physical denaturation.

11B. Pipet the DNase incubation mixture directly onto the center of HiBind™ RNA column silica membrane. Incubate at room temperature for 15 minutes.

11C. Pipet 400µl RNA wash Buffer I into the column and incubate at room temperature for 5 min. Centrifuge at 10,000 x g for 30 seconds. Discard the flow-through and collection tube.

12. Place the column into a new collection tube (provided). **Pipet 500 µl RNA Wash Buffer II (prediluted with absolute ethanol) into the column**. Centrifuge at 10,000 x g for 30 seconds. Discard the flow-through and re-use the collection tube.

    Note: RNA Wash Buffer II Concentrate must be diluted with absolute ethanol before use. Refer to label on bottle for instruction.

13. **Add another 500 µl RNA Wash Buffer II to the column and centrifuge at 10,000 x g for 1 minutes.** Discard the flow-through and re-use the collection tube.

14. Place the HiBind™ RNA Column into the same 2 ml collection tube. Centrifuge at full speed (≥12,000 x g) for 2 minutes to completely dry the membrane.

15. Elution of RNA: Place the HiBind® RNA column onto a 1.5ml RNase-free microtube and **add 30-50 µl DEPC-water directly onto the center of HiBind™ RNA column silica membrane**. Let the column sit at room temperature for 2 minutes and centrifuge at 10,000 x g for 1 minutes to elute RNA.

16. If the expected RNA is greater than 30µg, repeat the elution step (step 15) as described with a second volume of DEPC-treated water, collect the second elution with same collection tube. To obtain higher concentration of RNA, the second elution can be performed using the first eluate (from step 15).

To determine the concentration and purity of RNA, measure absorbency at 260 nm and 280 nm in a spectrophotometer. 1 O.D. unit measured at 260 nm corresponds to 44 µg of RNA per ml. If it is necessary to dilute RNA sample, use a buffer with neutral pH. The ratio of A_{260}/A_{280} of pure nucleic acids is 2.0, while for pure protein it is approximately 0.6. A ratio of 1.8-2.1 corresponds to 90%-100% pure nucleic acid. (Phenol has an absorbency maximum at 275 nm and can interfere with spectrophotometric analysis of DNA or RNA. However, the E-Z 96® RNA technology eliminates the use of phenol and avoids this problem.) Store RNA samples at -70°C in water. Under such conditions RNA is stable for more than a year.

Troubleshooting Guides

Problem	Cause	Suggestion
Little or no RNA eluted	RNA remains on the column	• Repeat elution. • Pre-heat DEPC-water to 70° C prior to elution. • Incubate for 5 min with water prior to elution
	Column is overloaded	• Reduce quantity of starting material.
Clogged collumn	Incomplete lysis	• Mix thoroughly after addition of TRK Lysis Buffer. • Reduce amount of starting material
Degraded RNA	Source	• Do not freeze and thaw sample more than once. • Follow protocol closely, and work quickly. • Low concentration of virus in the sample
	RNase contamination	• Ensure not to introduce RNase during the procedure. • Check buffers for RNase contamination.
Problem in downstream applications	Salt carry-over during elution	• Ensure RNA wash Buffer II has been diluted with 4 volumes of 100% ethanol as indicated on bottle. • RNA Wash Buffer II must be stored at room temperature. • Repeat wash with RNA wash Buffer II.
	Inhibitors of PCR	• Use less starting material • Prolong incubation with Buffer TRK to completely lyse cells
DNA contamination		• Digest with RNase-free DNase and inactivate at 75°C for 5 min.
Abnormal OD reading on A260/A280	DEPC residue remains in DEPC-water	• use different RNase-free water. • Remove DEPC by Autoclave • Use 10mM Tris-HCl, not the DEPC water to dilute the sample before measuring purity